University of Alberta

A structural analysis of convergent cleistothecial fungi representing the *Leotiomycetes* and *Sordariomycetes*

by

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Abstract

Pleuroascus nicholsonii and *Nigrosabulum globosum* were once placed among the *Pseudeurotiaceae* based on morphological characteristics, but molecular phylogenies indicated they are unrelated to each other and more likely affiliated with the *Leotiomycetes* and *Sordariomycetes*, respectively. A detailed reexamination of the morphology of *P. nicholsonii* showed that ascomata have dorsiventral polarity with appendages that arise basally or equatorially, and a thinning of subicular hyphae over the crown. Electron microscopy revealed that ascospores are uniseriate within a delimiting membrane system, and interascal sterile elements are paraphyses. These characters are compatible with the *Leotiomycetes*. *N. globosum* resembles perithecial hypocrealean relatives in that ascogenous filaments proliferate between cells derived from apical paraphyses. First-formed asci are clavate and those formed later are subglobose. These previously unexamined centrum characters support its affiliation with the *Sordariomycetes*. The cleistothecial form in these apothecial and perithecial lineages probably arose in response to the demands of a coprophilous lifestyle.

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Chapter 1

Introduction

This thesis describes the structural features underlying a profound convergence in ascomatal form in two distant lineages of *Ascomycota*, and focuses on the use of data from developmental morphology to support hypotheses about the ancestry and phylogenetic affiliations of representatives.

Most ascomata can be assigned to three structural types: Apothecia are saucer-shaped and have exposed hymenia; perithecia are flask-shaped and have partially enclosed hymenia, the products of which are released through an apical pore or ostiole; and cleistothecia are ball-shaped with hymenial tissues entirely enclosed. In many cases, this apparently crude categorization is taxonomically informative, especially when used in conjunction with morphological characters associated with the centrum which includes asci, ascospores and paraphyses, and their relationship to the sterile tissues surrounding and, or, supporting the hymenium.

Consistent ontogenetic patterns associated with the three types of ascomata have been used to refine classification schemes. For example, the origin of excipular and peridial tissues along with the development and organization of the centrum, i.e., the tissue giving rise to the ascogenous system within the ascomatal wall, have been a particularly significant source of phylogenetically valuable characters (Dangeard 1907; Nannfeldt 1932; Luttrell 1951; 1955; Cain 1956; Malloch 1981), and have been influential in identifying lineages within apothecial, perithecial and cleistothecial ascomycetes.

Molecular phylogenies have in many instances supported ascomycete classification schemes based on morphological data (e.g., Geiser & LoBuglio

2001; Zhang & Blackwell 2002; Hansen & Pfister 2006; Wang et al. 2006). They have also indicated where the morphological features of some species have been misinterpreted and/or where characters need closer scrutiny (e.g., Rehner & Samuels 1994; 1995; Rossman et al. 1999; Sugiyama et al. 1999; Suh & Blackwell 1999; Gernandt et al. 2001; Wang et al. 2006). Surprisingly though, there are relatively few studies that have re-examined such taxa or characters after DNA-based methods have indicated problems with classification. I have identified a need for these types of data and advance the position that careful re-examination of developmental morphology may not only indicate why taxa were originally misplaced and where morphological data were misinterpreted, but may also provide some basis for explaining the origins of convergent morphologies (Greif et al. 2004; Tsuneda & Currah 2004; Skinner et al. 2006; Greif & Currah 2007; Greif et al. 2007).

Here, I re-examine two cleistothecial ascomycetes, *Pleuroascus nicholsonii* Massee & Salmon and *Nigrosabulum globosum* Malloch & Cain that exhibit a striking convergence in ascomatal morphology and habitat. Both were placed relatively recently in the same family, i.e., the *Pseudeurotiaceae*. Although several more recent papers have suggested that they belong to quite different lineages, with *P. nicholsonii* in an apothecial lineage, and *N. globosum* most closely related to perithecial species, there has been no reanalysis of their morphological characters, or any attempt to explain the origin or basis of the convergent features of these two species.

***Pleuroascus nicholsonii* and *Nigrosabulum globosum* as cleistothecial ascomycetes**

Pleuroascus nicholsonii and *Nigrosabulum globosum* are similar in producing globose ascomata, i.e., cleistothecia, in which the asci are completely surrounded by sterile, darkly pigmented tissue made up of cells that appear polygonal in surface view. Asci appear to be distributed at random within the centrum and are short-lived or evanescent so that their globose, hyaline, single-celled ascospores are free and in no particular order when the fructification is mature. Sterile elements are intermixed among the ascospores in both species.

Historically, species with cleistothecia were placed among orders in the class *Plectomycetes* (Nannfeldt 1932). Patterns in the taxonomic occurrence of types of anamorphs (e.g., phialidic, annellidic, and arthroconidial), distinct developmental sequences associated with elements of the centrum, e.g., origin and disposition of paraphyses, other characters associated with ascomatal morphology (e.g., colour, texture, etc.), as well as ecological characteristics (e.g., preferences and predilections for habitats and substrates) eventually led to this taxon being dismantled.

Subsequent attempts at regrouping cleistothecial species and genera into phylogenetically more meaningful categories resulted in a variety of classification systems. Benny & Kimbrough (1980) provided a synopsis of the *Plectomycetes* basing their classification system on what they termed the "plectomycete centrum," i.e., globose, irregularly disposed, evanescent asci. Noting the heterogeneity of the class, they divided the *Plectomycetes* into six orders and twelve families. They acknowledged that there was sufficient evidence to support the hypothesis that the cleistothecium originated several

times in apothecial, perithecial, or pseudothecial lineages, and that several of their orders should be considered members of these groups. Von Arx (1987) suggested the *Eurotiales*, i.e., cleistothecial species with globose, evanescent, irregularly disposed, eight-spored asci, with single-celled ascospores, represented a single plectomycetous lineage. Using this broad concept, he divided the order into four families, the *Amauroascaceae* (containing *Pleuroascus*), *Eurotiaceae*, *Gymnoascaceae*, and *Onygenaceae*, on the basis of ascospore shape, size and symmetry. Although he espoused a more conservative estimate about the number of times the cleistothecium had originated within the *Ascomycota*, he offered that some cleistothecial taxa resembled those in apothecial and perithecial groups. He excluded the *Pseudeurotiaceae*, which he considered was probably "polyphyletic," from the *Eurotiales*.

Malloch & Cain (1970) erected the *Pseudeurotiaceae* to accommodate cleistothecial taxa that resembled species in the *Eurotiales* (*sensu* Fennell 1973) but had anamorphs in which phialoconidia are borne in sticky balls (e.g., *Acremonium*) rather than in chains (e.g., *Penicillium*), and darkly pigmented and occasionally lightly coloured peridia, irregularly disposed, globose to subglobose asci, and one- or two- celled, hyaline or brown, smooth or roughened aporate ascospores. In addition to *Pseudeurotium*, the family included *Cephalotheca, Emericellopsis*, *Fragosphaeria*, and *Testudinia,* along with five newly described genera, *Cryptendoxyla*, *Hapsidospora*, *Leuconeurospora, Mycoarachis*, and *Nigrosabulum*. In 1974, Malloch erected *Connersia* to accommodate *Pseudeurotium rilstonii*, and suggested its placement in the *Pseudeurotiaceae*. The family increased in size again in 1987 when von Arx included *Albertinia, Leucosphaerina* and *Pidoplichkoviella.*

Pleuroascus first appeared as a member of the *Pseudeurotiaceae* in 1995 (Hawksworth et al. 1995) although prior to this date it had been placed in the *Perisporaceae* (Massee & Salmon 1901), the *Onygenaceae* (Malloch & Benny 1973; Benny & Kimbrough 1980), the *Microascacae* (Lodha 1978), and the *Amauroascaceae* (von Arx 1987).

The polyphyletic nature of the *Pseudeurotiaceae* was suspected based on morphological and developmental features (e.g., Benny & Kimbrough 1980, Malloch 1981, 1987, von Arx 1987, Hawksworth 1994) and this suspicion was confirmed later in phylogenetic analyses by Rehner & Samuels (1994, 1995) and Rossman et al. (2001) using LSU rDNA, Gernandt et al. (2001), and Sogonov et al. (2005) using SSU rDNA, Suh & Blackwell (1999) using LSU and SSU, and Wang et al. (2006) using LSU + SSU + 5.8S rDNA. *Connersia*, *Pleuroascus*, *Leuconeurospora*, and *Pseudeurotium* formed a clade labelled "residual *Pseudeurotiaceae*" (Suh & Blackwell 1999) near the inoperculate discomycetes (*Leotiales*), a group of apothecial ascomycetes. *Bulbithecium*, *Emericellopsis*, *Hapsidospora*, *Leucosphaerina*, *Mycoarachis*, and *Nigrosabulum* constituted a cleistothecial clade among the perithecial *Bionectriaceae* (*Hypocreales*) (Suh & Blackwell 1999; Rossman et al. 2001). *Albertiniella*, *Cryptendoxyla*, and *Fragosphaeria (=Cephalotheca) sulfurea* showed affinity to the *Sordariales*, *Pidoplichkoviella* to the *Xylariales*, and *Fragosphaeria purpurea* to the *Ophiostomatales* (Suh & Blackwell 1999; Rossman et al. 2001). Putative relationships of cleistothecial taxa within apothecial and perithecial lineages warrants re-examination of these groups.

The apothecial ascomycetes

Apothecial ascomata in their simplest configuration resemble stalked or sessile cups or discs that support a layer of upright, cylindrical asci that forcibly discharge ascospores through an apical slit or pore (Cain 1956; Pfister & Kimbrough 2001). Several layers of prosenchymatous and/or pseudoparenchymatous cells form the excipulum tissue that supports the hymenium. Paraphyses interspersed among and parallel to the asci maintain the configuration of the hymenium while ascospores are discharged at various times (Cain 1956). The combination of exposed hymenium and forcible ascospore discharge are widely considered to be adaptations for wind dispersal of the meiotic products.

Most (non-lichenized) apothecium-forming ascomycetes are generally distributed among the genera of the *Pezizomycetes* and the *Leotiomycetes* and these classes are distinguished from each other in part on the basis of the ascus dehiscence mechanism. Ascus apices that are operculate are characteristic of the former and those with slit- or pore-like openings and without a lid, i.e., inoperculate, are characteristic of the latter.

Developmentally, these two groups are similar in that most species show an early "cleistohymenial" stage in which the ascogenous cells are entirely enclosed within a jacket of excipular tissue. As development proceeds, a small central opening appears on the upper surface that allows the excipulum to reflex and display the hymenial tissue (Kimbrough 1981).

The perithecial ascomycetes

There is a wide range in variation in the morphology of perithecial ascomata but

all consist of a globose, flask- or bottle-shaped structure that contains and protects the ascogenous system. Ascospores develop in cylindrical to club-shaped asci and are released through an ostiole, either through forcible ejection from the tips of asci that protrude through the ostiole at maturity and have a pore or slit at their apices, or through extrusion as a sticky mass after asci break down (Cain 1956; Pfister & Kimbrough 2001; Samuels & Blackwell 2001). Paraphyses or paraphysis-like structures are common in perithecial taxa. Apically derived paraphyses and periphyses that line the neck cavity also occur. Perithecia can also be immersed, whole or in part, in a loose to consolidated layer of vegetative hyphae that form a type of receptacle called a stroma.

Development in perithecial forms varies, but generally it resembles the sequence observed in the apothecial fungi in that there is an early cleistohymenial stage. However, development of the apical pore or ostiole in the ascoma is more complex and does not lead to the exposure of the entire hymenial layer. Instead, a tubular neck often generated by a meristematic zone, surmounts the more globose base of the ascoma and gives it the hallmark flask-shaped morphology (e.g., Goh & Hanlin 1994).

Thesis objectives

My hypothesis was that the ascomata of *Pleuroascus* and *Nigrosabulum* represent neotenous versions of developmental trajectories within normally apothecial and perithecial lineages that had been interrupted at a cleistohymenial stage. Consequently, I examined the development of their cleistothecia to search for convincing evidence of apothecial ancestry in *Pleuroascus*, and perithecial ancestry in *Nigrosabulum*, as indicated by published DNA-based phylogenies,

and to obtain data that would help explain the adaptive of significance of the gastroid ascomatal form in their respective habitats.

My investigations are organised into two chapters (i.e., chapters two and three). Chapter two examines the developmental sequence of *Pleuroascus nicholsonii* using light, scanning electron, and transmission electron microscopy. A similar approach was used to investigate the development of *Nigrosabulum globosum* in chapter three. In chapter four I summarize my findings, discuss the differences and similarities between these two ascomycetes, and review the relevance of developmental morphology to resolving problems in classification of these and related taxa.

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Chapter 2

Evidence of apothecial ancestry in the cleistothecial ascomata of *Pleuroascus nicholsonii* *

Introduction

Pleuroascus nicholsonii is a cleistothecial ascomycete described originally from guinea pig dung from Kew, England (Massee & Salmon 1901). The species is uncommon and only five additional collections, four of which are from rodent dung, have been reported, including CBS 389.78 from Argentina, CBS 110040 from Australia, UAMH 3979 from California (Malloch & Benny 1973), and H. JB-GM 2288 (Barrasa & Moreno 1984) and CBS 120410, a soil isolate, both from Spain. Despite these relatively few records, the species is significant because it may be phylogenetically close to other putative cleistothecial representatives of the inoperculate discomycetes, including *Connersia rilstonii*, *Leuconeurospora pulcherrima*, *Pseudeurotium zonatum*, (the so-called "residual *Pseudeurotiaceae*"), *Pseudogymnoascus roseus* and, more remotely, the *Myxotrichaceae*.

Previous descriptive accounts of *P. nicholsonii* provide few indications of apothecial ancestry or affiliation in this species and, in fact, treatments based solely on morphological data suggested its affinity would be with the perithecial or cleistothecial lineages. The globose, melanized cleistothecium of *P. nicholsonii* consists of an outer skin made up of a single layer of flattened, polygonal, melanized cells and an inner layer, several cells thick, of hyaline

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pseudoparenchyma. Long appendages of tightly coiled hyaline hyphae arise from the outer melanized layer. These characteristics were compatible with the taxon's placement in the *Perisporaceae* by Massee & Salmon (1901) and in the *Microascaceae* by Lodha (1978). Within the centrum and interspersed among the branches of the ascogenous system are sterile elements that disarticulate at maturity to form arthroconidium-like segments (Malloch & Benny 1973). These structures, along with the minute, single-celled, hyaline ascospores in globose, evanescent asci, were considered evidence supporting a relationship with onygenalean taxa (Malloch & Benny 1973). Later, the spherical, thick-walled ascospores led von Arx (1987) to place *P. nicholsonii* in the *Amauroascaceae*, a family in the *Eurotiales*.

A phylogenetic position among the inoperculate discomycete taxa, as indicated by DNA analyses (Suh & Blackwell 1999; Gernandt et al. 2001; Sogonov et al. 2005; Wang et al. 2006), suggested that a re-evaluation of morphological characters of *P. nicholsonii* and other "residual *Pseudeurotiaceae*" might show features derived from apothecial forerunners (Tsuneda & Currah 2004) and would thus provide a more detailed argument concerning the taxonomic relationships of these enigmatic species. Consequently, I used light and electron microscopy to revisit the morphology and development of the ascomata of *P. nicholsonii* and to lay the groundwork for further studies of related or similar cleistothecial taxa.

Materials and Methods

Pleuroascus nicholsonii (UAMH 3979) was grown on cornmeal agar (CMA, Acumedia, Baltimore, Maryland, USA), oatmeal agar (OA: 10 g oatmeal, 16 g

agar, 1 L dH_20), and tap water agar (TWA: 15 g agar, 1 L dH_20) at 20 °C under ambient lighting of the laboratory. Slide cultures were prepared using Pablum cereal agar (CER: 25 g Mead Johnson Mixed Cereal Pablum (Mead Johnson, Evansville, IN), 5 g agar, 250 ml dH_20) and TWA. Samples were examined and photographed with an Olympus BX50 microscope with an Olympus DP12 camera. Material for paraffin sectioning was obtained from 40-day-old cultures grown on CER, CMA, and OA and processed with a Fisher Histomatic Tissue Processor Model 160 (Fisher Scientific, Waltham, MA), embedded in paraffin, and sectioned at 6 µm. Sections were stained with Safranin and Fast green and mounted in DPX Mountant for Histology (Sigma Aldrich, St. Louis, MO).

For scanning electron microscopy (SEM), 5-mm disks of agar bearing ascomata at different ages were cut from mature colonies and fixed in unbuffered 2% glutaraldehyde for 4 h, placed in 2% tannic acid-2% guanidine hydrochloride overnight at 5 °C, and postfixed in 2% OsO_4 in phosphate buffer for 3 h at room temperature. Fixed material was dehydrated in an ethanol series, critical point dried in a Polaron E-3000 dryer using carbon dioxide, then coated with gold and examined at 15 kV with a Hitachi S-510 electron microscope. For transmission electron microscopy (TEM), specimens were fixed in 2% glutaraldehyde and 2% OsO_4 in phosphate buffer at pH 7.3 and dehydrated in an ethanol series. Samples were then embedded in Spurr's resin. Thin sections (0.5-1 µm) were stained with Toluidine Blue. Ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs were taken at 75 kV with a Hitachi H-7000 electron microscope.

Results

The first indications of ascoma formation were apparent within one week of inoculation and were single, short, hyaline recurved branches, approximately 5 μm across, that developed laterally on vegetative hyphae (Fig 2.1). Shortly afterwards, each initial was enveloped by hyphae that originated from the base of the branch, i.e., its origin, and from nearby vegetative hyphae, until a globose, knot-like structure (Figs 2.2-2.3) had formed. By the time these primordia were 20 μm in diameter, prosenchymatous hyphae were becoming septate and the outer layer was slightly melanized. Primordia increased to 110-330 μm in diameter as individual cells expanded 5-10 fold. Recruitment of hyphae toward the developing ascoma occurred throughout development but after the primordium had formed they were no longer integrated into the incipient peridium, creating instead a bramble-like layer of subicular hyphae enclosing the ascoma. This layer was thinnest, and sometimes discontinuous, over the crown of the cleistothecium (Figs 2.4-2.5).

As the ascoma matured further, the cells of the outermost layer increased in breadth so that they were 4-5 μm across, and formed a distinctive, heavily melanized epidermis consisting of more or less isodiametric and squamulose polygonal cells (Figs 2.5-2.6). Epidermal cells were delineated from adjacent cells in this layer by distinct fissures or grooves (Figs 2.5-2.8). Continued expansion of the ascoma resulted in the formation of a ridged border on the outer face of each cell that ran parallel to the intercellular fissures (Fig 2.5). In cross section, each cell in this layer, lacked cytoplasm at maturity, was rectangular, 2-4 μm thick, and had thickened, melanized outer tangential and radial walls, and a thinner, hyaline wall next to the underlying cells of the

peridium (Fig 2.6) that eventually disintegrated along with 3-4 layers of hyaline rectangular cells of the subepidermal layer. Physical disruption of the peridium (i.e., touching it with a probe) at this stage resulted in breaks that followed the grooves between cells, rather than tears across cells (Figs 2.7-2.8). Some epidermal cells, especially in the basal and equatorial portions of each ascoma, gave rise to septate hyaline hyphae 1.5-2.3 μm in diameter, that grew outwards in a tight helix consisting of up to 40 revolutions and up to 16.5 μm across, to form long, spring-like appendages (Fig 2.9). In some preparations, a groove ran the length of the hypha on the outer side of the helix (Fig 2.9). Cross sections of hyphae of the appendages indicated a pattern of wall thickening that positioned the narrowed lumen toward the outer margin of the helix (Fig 2.10).

Branched networks (Fig 2.11) of crosier-bearing hyphae (Figs 2.11-2.12) arose from the inner surface of the subepidermal layer producing asci in a sympodial pattern. Mature asci were 8-spored, 7-8 μm in diameter and extended in columns into the centrum (Figs 2.15-2.16). Asci detached from the supporting hyphae at an early stage in development and soon afterwards the ascus wall was scarcely detectable (Figs 2.13-2.14). At maturity, ascospores were hyaline to pale pink, globose, and conglobate in petalloid, decussate, or curvilinear ascal clusters (Figs 2.15-2.16, 2.19-2.20). Ascospores were 2-3.8 μm in diameter and had refractive walls (Fig 2.19) that were mostly smooth although minute amounts of an amorphous material adhered to the surface of some (Fig 2.20). TEM showed that the ascospore wall was 0.5 μm thick, and multilaminate with a thick inner electron-lucent layer surrounded by a thinner but more electron dense region (Figs 2.21-2.22). Ascospore contents were strongly osmiophilic. By TEM, conglobate ascospores appeared as short, c-shaped chains, some of

which appeared to be enclosed within an osmiophilic, membrane-like sleeve (Fig 2.22). When present, this sleeve was slightly irregular in thickness and closely followed the contours of each chain, invaginating sharply at the junctions between adjacent ascospores. In some sections, abutment zones between adjacent catenate ascospores were traversed by two bands of electron lucent wall material, which were continuous from one spore to the next (Figs 2.21-2.22).

Sterile filaments were prominent components of the centrum and at maturity consisted of branched or unbranched chains of easily disarticulated, thick-walled cells, 3-8 x 7-11 μm, with navicular to fusiform basal segments and bulbous, irregularly swollen tips (Figs 2.17-2.18). By TEM, these contained cytoplasm (Fig 2.17) and had angular deposits of an electron dense material on the outer wall surface (Fig 2.22).

Within two weeks of inoculation, numerous clusters of dark brown to black ascomata were surrounded by a hyaline subicular layer that generally became buff to pink before collapsing and leaving the ascomata partially supported by the interlacing helical appendages (Fig 2.4). Mature ascospores were refractive and formed a dry, powdery mass within the peridium. When placed on water agar, ascospores swelled to approximately 5 μm in diameter and formed a single germ tube within 24 hrs.

Discussion

My objective in reexamining the structure and development of the cleistothecial ascomata of *Pleuroascus nicholsonii* was to find anatomical features that would support results from analyses of DNA sequence data that indicate apothecial

ancestry. My observations agree generally with previous accounts that had been based on examinations of squash mounts with the light microscope (Massee & Salmon 1901; Malloch & Benny 1973; Barrasa & Moreno 1984) but, because I used a combination of thin-sectioning and both TEM and SEM, some significant new perspectives were obtained.

The early stages of ascoma initiation in *P. nicholsonii* are signaled by the formation of small, recurved hyphal branches or initials. These provide foci for enveloping hyphae that lead to the formation of primordia. Subsequent septation, especially in hyphae of the outer layers where melanization takes place rapidly early in development, leads to the formation of a stratified peridium consisting of an epidermal layer of darkly pigmented, flattened cells and a hyaline subepidermal layer several cells thick. This stratification was also noted by Malloch & Benny (1973). The unusual pattern of wall thickening and melanin deposition in the outer and radial walls of the epidermal cells creates a layer that is distinctly squamulose and allows the mature peridium to come apart through the lines of weakness that occur along intercellular junctions. This configuration is reminiscent of cephalothecoid peridia exemplified by *Chaetomidium leptoderma* and *Cryptendoxyla hypophloia*. However, in these sordariaceous cleistothecial taxa, plates made up of radiating hyphae break apart through predetermined lines of weakness that develop across rather than in between cells (Greif et al. 2004; Greif & Currah 2007). A similar construction is described for *Leuconeurospora pulcherrima,* also of the *Pseudeurotiaceae* (Malloch & Cain 1970), but in ruptured peridia of *P. nicholsonii,* and the similar taxon *Connersia rilstonii* (Malloch 1974), plates appear to be composed of random clusters or patches of isodiametric cells. How such a configuration of

peridial cells might contribute to reproductive fitness is unknown but ascospore release appears to require some degree of ascomatal disruption or displacement, possibly abetted in *P. nicholsonii* by the tangled elastic helical appendages (see below). An extreme variant of the pattern of epidermal cell maturation observed in *P. nicholsonii* is represented by the heavily melanized peridial elements of the cleistothecium-like apothecium of *Myxotrichum* (Tsuneda & Currah 2004). In this species and others in the genus, incipient peridial elements are free from each other, even at the primordium stage and remain so as they become heavily melanized to form a structure that can be impaled on the setae of passing microfauna (Greif & Currah 2003). The peridial elements of *Pseudogymnoascus roseus*, a taxon that is phylogenetically closer to *Pleuroascus nicholsonii* (Sugiyama et al. 1999; Mori et al. 2000; Jiang & Yao 2005) are comparable to those of *Myxotrichum* but lack melanization (Tsuneda 1982; Jiang & Yao 2005).

My observations of the production of globose to subglobose asci from crosiers on a branched ascogenous system within the centrum of *P. nicholsonii* are also consistent with prior accounts (Massee & Salmon 1901; Malloch & Benny 1973; Barrasa & Moreno 1984). Asci arise sympodially and in files along branched ascogenous axes that have a basal crosier, and mature more or less synchronously. A similar pattern is seen in the closely related taxon *Connersia rilstonii* (Suh & Blackwell 1999; Gernandt et al. 2001) in which a similar arrangement results in the appearance of asci being produced in helicoid chains (Malloch 1974).

My TEM observations of ascus morphology in *P. nicholsonii* indicate that the apparently globose asci have a unique and hitherto unreported structural configuration that is a strong indicator of the species' apothecial ancestry. The

electron-dense sleeve enveloping a uniseriate arrangement of ascospores is interpreted here as the vestiges of the outer, or spore-investing, membrane of a delimiting membrane system (Read & Beckett 1996). This interpretation is supported by the pronounced invaginations of this sleeve-like structure at the points of contact between adjacent ascospores and by the narrow cavity that occurs between spores in the chain that is either lined or filled with similar electron dense material. The latter configuration ostensibly represents a portion of the investing membrane trapped between developing ascospores, which appear to be separated by a ring of electron-lucent material similar, or identical to, ascospore wall constituents. When young and still attached to ascogenous hyphae, asci were more or less globose and appeared to be typically prototunicate. My observations indicate that the typical decussate to curvilinear arrangement of ascospores observed in squash mounts using light microscopy arises from the curling and compacting of a cylindrical enveloping membrane system within the ascus wall (Fig 2.23). With no role in the forcible liberation of ascospores, asci evanesce at an early stage, leaving the ordered but twisted files of ascospores free within the ascoma.

In cross section, ascospores have a thick, bilaminate wall made up of a material that is electron lucent compared to the relatively electron-dense cytoplasmic contents. This cross-sectional morphology bears a strong resemblance to the ascospore walls of *Myxotrichum arcticum* (*Leotiomycetes*) which are also thick, multilaminate and electron lucent. In contrast, cross sectional profiles of the ascospores of eurotiomycete taxa (e.g., *Auxarthron* and *Arthroderma*) differ in having more distinct but thinner strata (Skinner et al.

2006; Tanaka et al. 1989). Ascospores also appear to be randomly dispersed in the ascus, rather than ordered, as in *P. nicholsonii.*

The abundant, thick-walled sterile elements interspersed among the asci have been interpreted as the conidiogenous precursors of an arthroconidial state (Malloch & Benny 1973; Barrasa & Moreno 1984). At maturity, these filaments disarticulate at the septa and superficially resemble arthroconidia. Cleistothecia or cleistothecium-like structures bearing conidia in the centrum are uncommon but do occur among the *Onygenales* (Sigler & Carmichael 1976) and in sterile *Myxotrichum*-like gymnothecia produced by *Oidiodendron maius* (Rice & Currah 2002). In these taxa, arthroconidia are also produced on hyphae external to the ascomata whereas a distinct conidial state is unknown in *P. nicholsonii*. However, a more plausible interpretation is that these structures in *P. nicholsonii* represent paraphyses. These sterile filaments, that extend from the subhymenium and stand parallel to asci in apothecial taxa, often have swollen tips and can also be branched and septate. In *P. nicholsonii*, the sterile filaments originate from the hyphae lining the inner wall of the peridium and then extend into the centrum where they exhibit distinct polarity, being made up of one or more elongate cells crowned by short, swollen branches at the apex. Tsuneda & Currah (2004) found that short, unbranched paraphyses were common among the globose asci of *Myxotrichum arcticum*, a more or less cleistothecial species that still retains significant vestiges of a flat hymenial layer. In *P. nicholsonii*, the filaments may function in a way similar to the capillitia in gasteromycetes, e.g. *Lycoperdon* or *Bovista*, by supporting the thin wall of the ascoma as the ascospores are liberated through cracks in the peridium.

The unilateral thickening on the spiral appendages noted by Malloch & Benny (1973) occurs along the inner wall of the tightly wound hyphae and the lumen is offset toward the outer side of the helix. In some of the material prepared for the SEM, the thinner wall on the outer face of the helix appeared to be pulled inwards, forming a groove or invagination running the length of the helix. The differential pattern of wall thickening would maintain the curvature of the helices and contribute to their characteristic elasticity. Most of the appendages on a single ascoma originate from peridial cells located from the base to the equatorial region, a feature noted in Massee & Salmon's original description (Massee & Salmon 1901). On agar media, appendages of adjacent ascomata become entangled so that large numbers of fruit bodies adhere to one another thus forming a large interconnected mass after the colony matures and the vegetative hyphae senesce.

I hypothesize that gasteromycetation developed in the apothecial predecessors of *P. nicholsonii* as a consequence of the transition from an association with plants, the usual substrates for *Leotiomycetes*, to a coprophilous habit. During ascoma differentiation in the inoperculate discomycetes, the hymenial layer arises within a cavity enclosed by a jacket of sterile tissue. As the young ascoma matures, the roof of the cavity thins toward the centre eventually pulling apart to expose the underlying asci (Bellemère 1967). During the evolution of *P. nicholsonii*, this developmental trajectory may have been interrupted so that the hymenial layer was not exposed. This may confer a selective advantage by keeping the minute, lightly pigmented meiotic propagules protected within the jacket of excipular tissues. Melanization of the outer layer of the jacket would make this strategy even more effective. With

closure, the entire ascoma with its mass of ascospores may become the dispersal unit, in which case the spring-like appendages that bind clusters of ascomata together may also function as attachment or disruption devices that facilitate transport and/or dispersal by small animals (e.g. insects and other arthropods), especially those that visit rodent dung. The thick-walled ascospores that germinate easily without any proxy treatment for gut passage would seem to indicate that this fungus probably does not rely on ingestion by a vertebrate carrier.

While the steps leading to the selection of the cleistothecial habit remain speculative, analyses of rDNA data have shown that the relationships of taxa within the *Pseudeurotiaceae* and the *Myxotrichaceae* need reconsideration (Suh & Blackwell 1999; Wang et al. 2006). My observations here suggest that a closer examination of structure and development in other gastroid *Leotiomycetes* could yield additional data for realigning the genera in these families and for rationalizing their ecological characteristics and taxonomic relationships.

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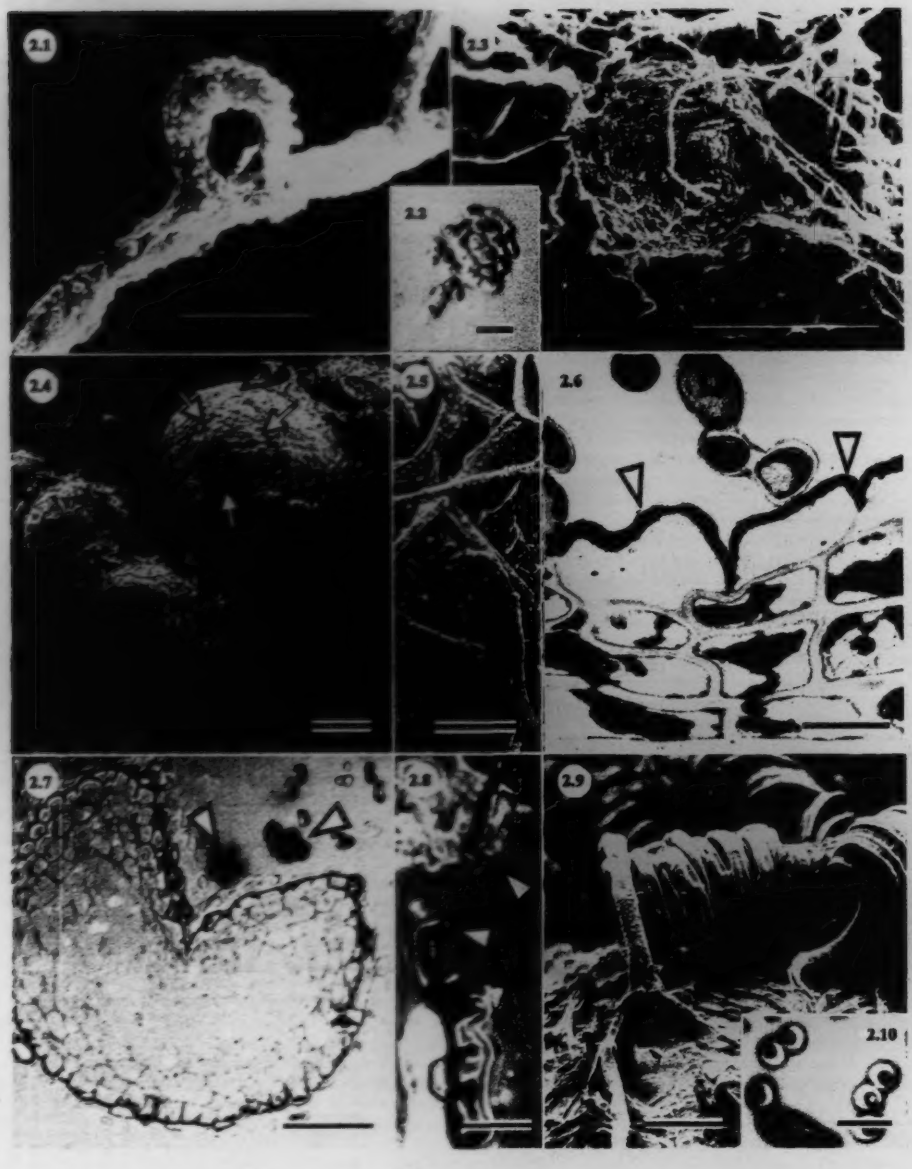
2.7. Light micrograph of thick section of peridal wall of mature ruptured ascoma with decussate and curvilinear asci (arrowheads). Bar = 10 μm.

2.8. Light micrograph of thick section of ascoma showing breaks between cells. Arrowheads indicate walls of a pair of previously contiguous cells. Bar = 5 μm.

2.9. Scanning electron micrograph showing spring-like appendages arising from epidermis. A groove runs along the length of the outer side of the helix. Bar = 10 μm.

2.10. Light micrograph of thick section through helical appendage showing the pattern of wall thickening with the narrowed lumen positioned towards the outer margin of the helix. Bar = 4 μm.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Figs. 2.11-2.16. Ascosporogenesis in *Pleuroascus nicholsonii* (UAMH 3979).

2.11. Light micrograph showing immature asci being produced in a sympodial pattern from a branched hypha. Bar = 5 μm.

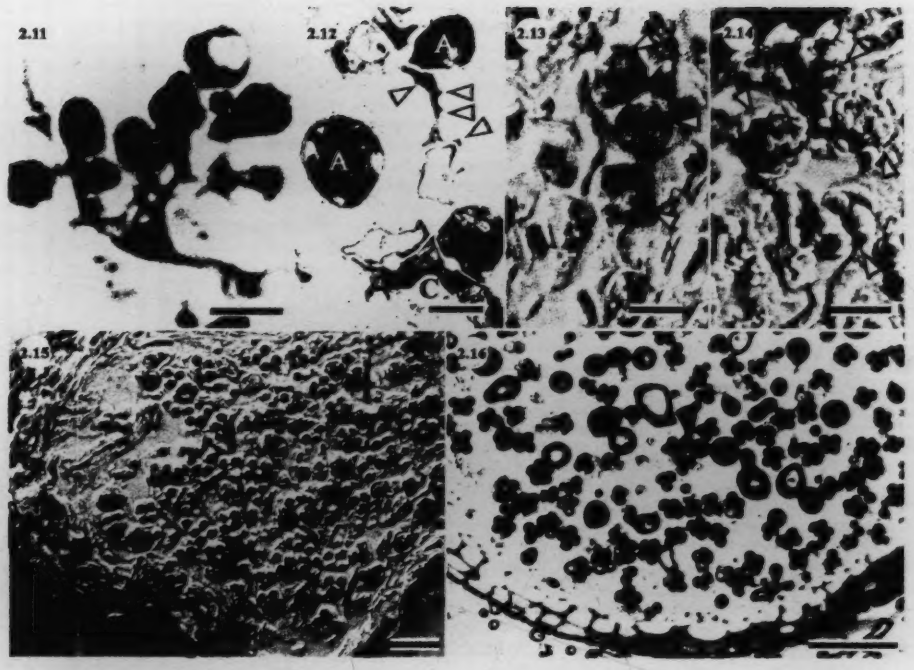
2.12. Transmission electron micrograph showing asci (A), crozier (C), and the branched ascogenous system. Note the basal stumps indicating the position of detached asci (arrowheads). Bar = 2 μm.

2.13. Thin section showing an early stage in ascus formation. Asci indicated by arrowheads. Bar = 5 μm.

2.14. Thin section showing a slightly later stage of ascus formation. Asci indicated by arrowhead. In both Figs 2.12-2.13 note the sympodial arrangement of asci. Bar = 5 μm.

2.15. Light micrograph of thin section of the ascoma. The branched network of crozier-bearing hyphae arise from the inner surface of the peridial wall to fill the centrum. Bar = 20 μm.

2.16. Thin section of mature centrum. Note asci in short chains and swollen sterile elements. Bar = 20 μm.


2.11
2.12
A
A
C
2.13
2.14
2.15
2.16
D

Figs 2.17-2.22. Morphology of asci and sterile elements in the mature centrum of *Pleuroascus nicholsonii* (UAMH 3979).

2.17. Transmission electron micrograph showing disarticulation of sterile elements in mature centrum. Arrow indicates point of fragmentation. Bar = 6 μm.

2.18. Thin section showing sterile filaments, arising from the inner surface of the peridial wall, consisting of navicular to fusiform basal segments and bulbous, irregularly swollen tips. Bar = 5 μm.

2.19. Light micrograph of globose to subglobose asci. Mature ascospores in petalloid (P), descussate (D), or curvilinear (C) clusters. Bar = 6 μm.

2.20. Scanning electron micrograph of a mature conglobate cluster of ascospores. Ascospore walls are smooth with minute amounts of amorphous material adhering to the surface. Bar = 2 μm.

2.21. Transmission electron micrograph of ascospores showing thick, electron lucent, multilaminate wall and osmiophilic contents. Bar = 2 μm.

2.22. Transmission electron micrograph of ascospores that appeared in short c-shaped chains, some of which appear enclosed within an osmiophilic, membrane-like sleeve (arrows). In some sections abutment zones between adjacent catenate ascospores are traversed by two bands of electron lucent wall material which are continuous from one spore to the next (arrowheads). Angular deposits of electron dense material are present on the outer surface of a sterile element (double arrowhead). Bar = 2 μm.

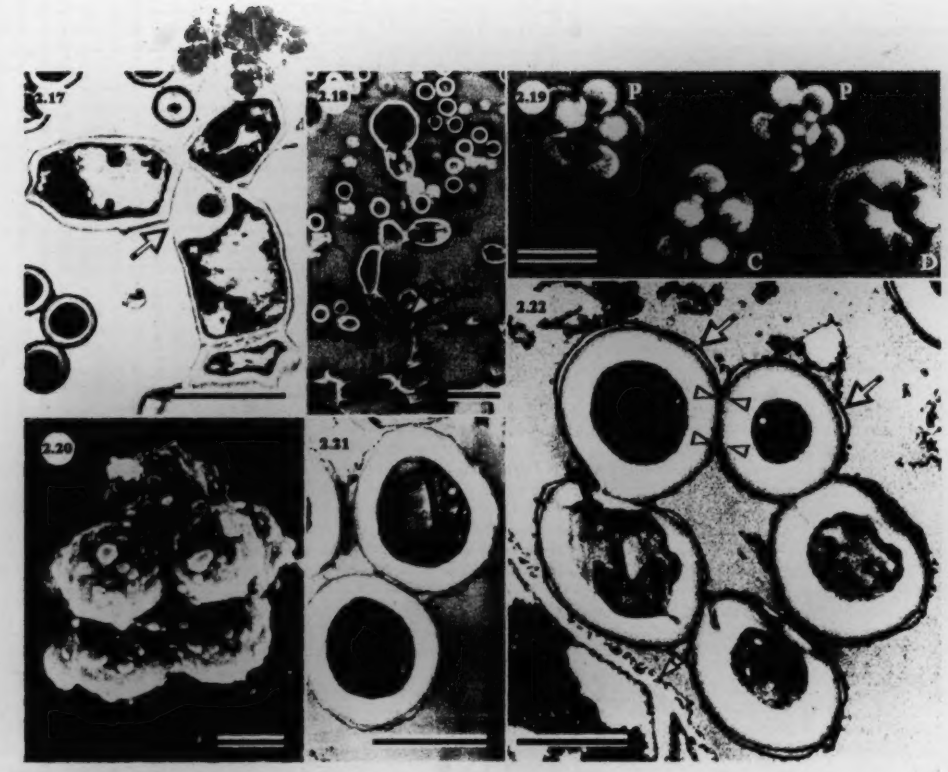
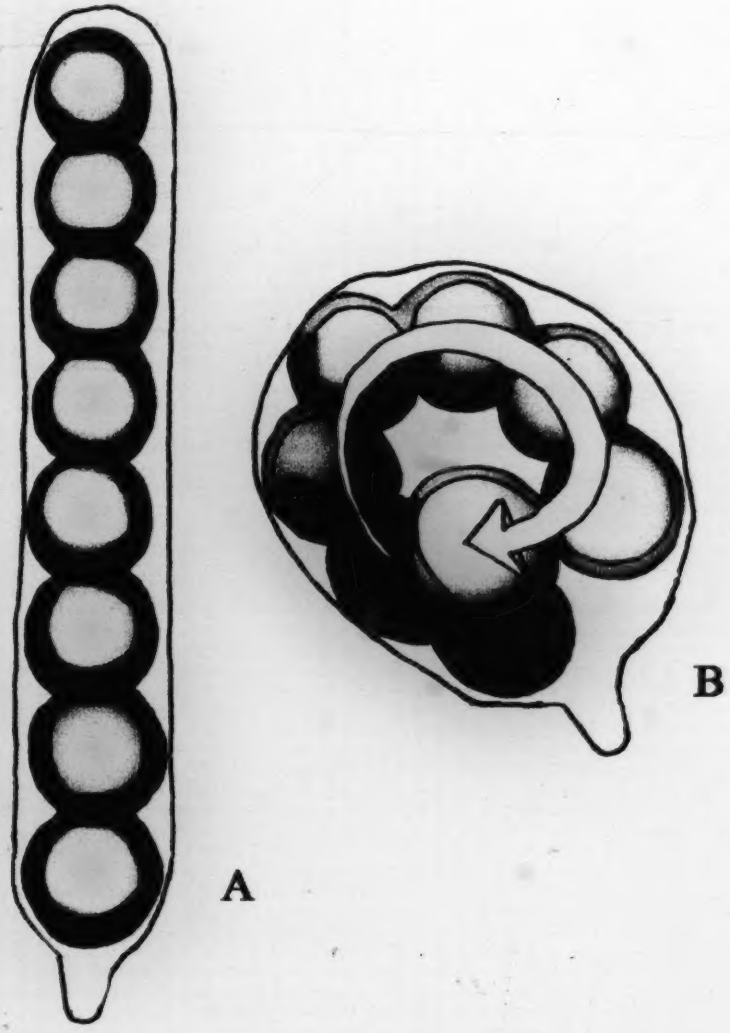

2.17
2.18
2.19
P
P
C
D
2.20
2.21
2.22

Fig 2.23. Comparison between a clavate ascus precursor and the globose ascus of *Pleuroascus nicholsonii.*

2.23 A. Ordered, uniseriate ascospores within a clavate ascus surrounded by the enveloping membrane system, typical of inoperculate discomycetes.

2.23 B. Ordered ascospores within a curling, enveloping membrane system and surrounded by a globose ascus wall, as exemplified by *P. nicholsonii*.

2.23



Chapter 3

Morphology and development of *Nigrosabulum globosum*, a cleistothecial coprophile in the *Bionectriaceae* (*Hypocreales*) †

Introduction

The *Pseudeurotiaceae* was erected to accommodate cleistothecial genera having irregularly disposed, evanescent asci and small, aporate ascospores (Malloch & Cain 1970). Results of molecular phylogenetic analyses (Rossman et al. 1999; Suh & Blackwell 1999; Rossman et al. 2001), supported in some cases by detailed morphological studies (Greif et al. 2004; Plishka et al. 2008), showed that the family was heterogenous and included taxa allied to the *Leotiomycetes* (e.g., *Pseudeurotium* and *Pleuroascus*), the *Sordariomycetes* (e.g., *Cryptendoxyla*), and the *Hypocreales* (e.g., *Nigrosabulum*). I recently reexamined an isolate of *Nigrosabulum globosum* that had been obtained from carnivore dung (coyote) collected in Alberta. A comparison of the SSU rDNA sequences (1645 bp, Appendix D) of this new isolate with GenBank AF096180, from the ex-type strain (M.J.R.P., unpubl.), showed 98% similarity and supported our identification, at least to genus. However, I found it difficult to reconcile the morphology of the mature ascomata of this species with a putative position among the *Hypocreales*. In this order, most species are perithecial and saprobic or parasitic on plants, fungi, etc., rather than cleistothecial and coprophilous.

Since its original description, *N. globosum* has been reported primarily

† A version of this chapter will be submitted to Mycological Research as: Plishka MJR, Tsuneda A, Currah RS, 2008. Morphology and development of *Nigrosabulum globosum*, a cleistothecial coprophile in the *Bionectriaceae* (*Hypocreales*).

from dung, and from Australia, Europe, Africa and in North America. Ascomatal structure and morphology, as portrayed in the line drawings included in the description of the type species, provide few hints that would indicate a relationship to the *Hypocreales* (Malloch & Cain 1970), and detailed studies of its development, beyond the brief analysis accompanying the original description, have not been done.

Hypocrealean fungi exhibit a distinctive pattern of centrum development that involves the formation of cylindrical asci that grow up through a network of apical paraphyses that may disintegrate at maturity. These features are not evident in squash mounts of cleistothecia of *N. globosum*, which show only that the bilayered ascoma wall surrounds a mass of globose, apparently unordered ascospores, each often having one or two blunt protuberances. Neither asci nor interascal tissue can be discerned reliably in these preparations, even when younger specimens are examined.

Considering the putative phylogenetic position of *N. globosum* among the *Hypocreales*, I anticipated that centrum development in the cleistothecia of this fungus should follow a pattern typical of the order. Using a combination of light and electron microscopy I examined stages in the formation of mature ascomata to derive data that could be used to test this hypothesis.

Materials and methods

Nigrosabulum globosum (UAMH 4433) was grown on cornmeal agar (CMA, Acumedia, Baltimore, MD), oatmeal agar (OA: 10 g oatmeal, 16 g agar, 1 L dH_20) and tap water agar (TWA: 15 g agar, 1 L dH_20) at 20 °C under ambient lighting of the laboratory. Slide cultures were prepared using Pablum cereal agar

(CER: 25 g Mead Johnson Mixed Cereal Pablum (Mead Johnson, Evansville, IN), 5 g agar, 250 ml dH_20) and TWA. Samples were examined and photographed with an Olympus BX50 microscope with an Olympus DP12 camera. For fluorescence microscopy, material was stained with 0.5 μg/mL Hoechst 33258 (Sigma-Aldrich, Canada), a bisbenzimide DNA intercalator that excites in the near UV and emits in the blue region, in buffered saline (1.37 M NaCl, 36 mM KCl, 100 mM Na_2HPO_4, 17.6 mM KH_2PO_4, pH 7.4), sealed under a coverslip with nail polish and kept at 5°C for 6 hours before viewing with a Leica DMRXA fluorescence microscope with a Leica HCX PL Fluotar 100X/ 1.30 oil immersion objective. Images were prepared with an Optronics DXM digital camera using UV laser illumination with emission filters set at 425-475 nm (blue region).

For scanning electron microscopy (SEM), 5-mm disks of agar bearing ascomata at different ages were cut from mature colonies and fixed in unbuffered 2% glutaraldehyde for 4 h, placed in 2% tannic acid-2% guanidine hydrochloride overnight at 5 °C, and postfixed in 2% OsO_4 for 3 h at room temperature. Fixed material was dehydrated in an ethanol series, critical point dried in a Polaron E-3000 dryer using carbon dioxide, then coated with gold and examined at 10 kV with a Hitachi S-510 electron microscope. For transmission electron microscopy (TEM), and thin-sections observed by light microscopy (LM), specimens were fixed in 2% glutaraldehyde and 2% OsO_4 in phosphate buffer at pH 7.3 and dehydrated in an ethanol series. Samples were then embedded in Spurr's resin. Thin sections (0.5 -1 μm) were stained with Toluidine Blue and examined using the Olympus BX50 microscope (see above). Ultrathin sections were stained with uranyl acetate and lead citrate.

Photomicrographs were taken at 75 kV with a Hitachi H-7000 electron microscope.

Results

When transferred to CMA or OA, globose ascospores, 2.5-5.0 μm in diameter, swelled and produced 1-3 germ tubes within 36 hours (Fig 3.1). After 14 days at 22 °C on these media, colonies were glabrous to sparsely tufted and hyaline toward the periphery, but olive green to charcoal towards the centre where dense, aggregations of grit-like, melanized cleistothecia had formed. Initiation of ascomata was first evident by the formation of sessile to stalked, globose, lateral branches on prostrate vegetative hyphae (Fig 3.2). Subsequently, many of these initials were each encircled by a swollen septate hypha (Fig 3.3) which then formed lateral buds that proliferated, branched and arched over the enlarging primordium (Figs 3.4, 3.6). Relatively broad hyphae arising from neighbouring vegetative mycelium (Fig 3.5) were also incorporated into the outer layers of the primordium. As this structure continued to increase in diameter, hyphae toward the periphery became closely septate and formed a pseudoparenchymatous tissue of cells, 2-4 layers deep, with thick melanized walls, while thin-walled cells towards the interior swelled to fill the centrum (Figs 3.6-3.7), became vacuolated and deliquesced, leaving an amorphous gel containing numerous pale yellowish, osmiophilic globules. A few intact and densely cytoplasmic cells on one side of the centrum (Figs 3.8) developed into hyaline branching hyphae that became thick-walled and formed a prosenchyma, 2-4(6) layers thick, lining the peridium. A second prosenchyma, of relatively thin-walled cells, then arose from the opposite pole and filled the centrum with a

tissue composed of tightly packed, more or less parallel, hyaline hyphae that were irregularly swollen between the septa (Figs 3.9-3.10).

Densely cytoplasmic ascogenous hyphae grew in through this prosenchyma and formed cylindrical to clavate asci with eight ascospores that often appeared to be uni- or bi-seriate in arrangement (Figs 3.11-3.13, 3.15). As ascogenesis proceeded, asci became more irregular or distorted in shape with some eventually being globose to subglobose (Figs 3.11, 3.13, 3.15). With further maturation, asci and most of the peripheral and interascal prosenchyma tissues within the thick-walled melanized outer cells of the ascoma deliquesced leaving only a few remnants along with traces of ascus wall material and delimiting membranes (Figs 3.14-3.16, 3.18-3.19).

By SEM and TEM, ascospores were globose to polyhedral, up to 5.8 μm in diameter and with thick (0.1- 0.6 μm) electron lucent walls, and strongly osmiophilic contents (Figs 3.14-3.16). By LM, ascospores appeared smooth but both SEM and TEM images showed a honeycomb-like network of ridges that appeared to interdigitate between adjacent ascospores (Figs 3.14-3.16). Fluorescence staining showed that ascospores each had two nuclei, both positioned towards one side of the spore (Fig 3.17). By LM, some mature ascospores had one or two papilla-like structures protruding from the surface. Sections of older cleistothecia showed ascospores with well developed germ tubes that had grown into the remnants of tissue lining the centrum and the peridium (Figs 3.18-3.19). Remains of paraphyses were sparse but evident among the ascospores (Fig 3.14, 3.18-3.19).

Discussion

Even though the mature, darkly pigmented, globose cleistothecia of *Nigrosabulum globosum* outwardly provide few structural clues to a putative ancestry among the *Hypocreales*, patterns of development associated with the centrum are compatible in most respects with perithecial representatives of this order. Ascomatal initials were formed by the production of bulbous lateral branches that were encircled by swollen septate hyphae. We interpreted these as a central antheridium and an encircling ascogonium. These paired gametangia were foci for the development of concentric masses of hyphae that arose from the ascogonium or its immediate vicinity. Similar initials occur in *Bulbithecium hyalosporum* (Udagawa & Muroi 1990), a monotypic genus phylogenetically close to *N. globosum* (Suh & Blackwell 1999; Rossman et al. 2001).

As primordia differentiated they passed through a stage in which central, lipid-rich cells disintegrated to form a gelatinous matrix that was subsequently replaced, first by basally derived peridial tissue, and second by a dense mass of apically derived paraphyses that grew downwards to fill the centrum with a thin-walled prosenchyma. This preascogenous pattern of development also occurs in *Bionectria ochroleuca*, and *Hydropisphaera peziza* (Hanlin 1961, 1963; as *Nectria gliocladioides* and *Neuronectria peziza* respectively) both now assigned to the *Bionectriaceae* (Suh & Blackwell 1999; Rossman et al. 2001).

While the central cells of the primordium were breaking down, cells derived from vegetative hyphae making up the outer wall of the ascoma became thick-walled and melanized. This outer layer, which eventually became carbonaceous, appears to represent stromatic tissue that at later stages clearly surrounded a hyaline peridium of basally derived, thick-walled prosenchyma.

These two distinct strata, i.e., the dark outer pseudoparenchyma and the hyaline inner prosenchyma, were considered originally to be constituents of a bilayered peridium (Malloch & Cain 1970), but our observations of development, and the putative position of *N. globosum* among the *Bionectriaceae* (Rossman et al. 2001), strongly suggests that the cleistothecium represents a uniloculate stroma that encloses an ascoma with a distinct peridium. The stromatal nature of a similar dark outer layer was suggested by Hanlin (1961) in the related but ostiolate species *Bionectria ochroleuca*. A similar interpretation may also be applicable to the melanized outer wall layers of the ascomata of *Hapsidospora irregularis*, a bionectriaceous genus also placed originally in the *Pseudeurotiaceae*, and to *Bulbithecium hyalosporum*. The hyaline peridium in *N. globosum* further supports its affiliation with the *Hypocreales* which usually have light to brightly colored, rather than melanized, peridia.

Malloch & Cain (1970) depict asci of *N. globosum* originating in the absence of crosiers in an almost palmate cluster at the apex of an ascogenous hypha. From our observation, deeply staining ascogenous hyphae presumably arose from the basal portion of the ascoma in *N. globosum* and grew in between the paraphyses. Asci appeared to form in clusters at the apices of ascogenous hyphae with some elongating to become cylindrical to clavate and others remaining globose, possibly because space constraints within the crowded centrum. In some cases, asci were so closely packed together that they appeared to have formed in short chains. Wu & Kimbrough (1990) interpreted their TEM sections of *Emericellopsis microspora*, a closely related genus in the *Bionectriaceae*, to indicate that asci arose through "repetitive generation from crosiers." We did not see crosiers in *N. globosum* but the formation of asci in

clusters near the apices of ascogenous hyphae would explain why some asci appeared butted up together in chain-like configurations.

Deliquescence of the asci left the ascospores free within the centrum, where they were intermingled with remains of the paraphyses. When squashed, mature cleistothecia did not release intact asci, although ascospores often appeared in pairs, triplets or in short chains of three (or rarely four), suggesting the presence of connecting material or mechanism. SEM and TEM preparations showed that the ascospore wall was thick, electron lucent and had a network of ridges and pits or grooves that interdigitated between adjacent ascospores and these interlocking configurations may have been sufficient to keep adjacent spores attached to each other. Low, to pronounced and wing-like ridges, as in *H. irregularis* and *E. microspora*, respectively, are common in ascospores of the *Bionectriaceae* (Hanlin 1963; Malloch & Cain 1970; Hanlin 1971; Wu & Kimbrough 1990) and have also been observed to interdigitate when examined in TEM sections (Wu & Kimbrough 1990). The papillate protuberances on some ascospores of *N. globosum*, observed here and by Malloch & Cain (1970) as "small particle-like attachments", are most likely the first indications of the formation of germ tubes that were able to extend into the tissues of the peridium and surrounding stroma (Figs 3.18-3.19). Germination *in situ* at least indicates that gut-passage is not a required step in the dissemination of this coprophile, and that an alternative explanation for dispersal must be found.

The homothallic character of *N. globosum*, noted in cultures used to prepare the original description (Malloch & Cain 1970), is possibly due to the binucleate status of the ascospores. Secondary homothallism, i.e., where each nucleus of the pair carries an opposite mating type factor, may explain why

paired gametangia precedes the formation of ascomata.

Malloch & Cain (1970) noted that a conidial state, with single, long, tapering conidiophores bearing ellipsoid, fusoid, ovoid or allantoid phialospores in moist clusters, was associated with their material, but an anamorph was absent in UAMH 4433 both during our study, and when it was originally isolated in 1981. Phialidic anamorphs, such as this typical *Acremonium* state are well known in the *Hypocreales* and particularly in the *Bionectriaceae*.

My data clearly show that the development of ascomata in *N. globosum* fits the pattern established for the *Hypocreales* and support the hypothesis, advanced on the basis of DNA sequence similarity, that there is a close relationship between this and several other cleistothecial genera in the *Bionectriaceae*. For instance, we show that the ascospores of *N. globosum* are not smooth, as originally described (Malloch & Cain 1970), but ornamented with a distinctive reticulum that bears a strong resemblance to the pronounced net-like pattern of ridges on the ascospores of *Hapsidospora*. Line drawings included with Malloch & Cain's (1970) original description of *H. irregularis*, and illustrations accompanying the description of *H. milkoi* (Beliakova 1975), show that the ascomatal wall also comprises two layers: the outer one dark and stromatic and the inner one hyaline and presumably representing peridial tissue. These teleomorphic similarities, along with its putative *Acremonium* anamorph, suggest *N. globosum* and the two species of *Hapsidospora* could possibly be considered congeneric. *Bulbithecium hyalosporum* is a monotypic cleistothecial genus that also shows strong morphological similarities to *N. globosum* in having a similarly constructed bi-layered ascoma wall and an *Acremonium* anamorph. Ascospores were described as smooth by LM (Udagawa & Muroi

1990), but might prove to be reticulate if examined by SEM.

There appear to be fewer morphological similarities between these three genera and *Mycoarachis*, which has been shown to cluster with them in a weakly supported clade (Suh & Blackwell 1999; Rossman et al. 2001). *Mycoarachis inversa*, which was also described with an *Acremonium* state, has smooth, two-celled ascospores and an ascoma wall that is bilayered, but in this case the outer layer is hyaline and the inner one is melanized (Malloch & Cain 1970). A detailed analysis of ascoma development, and a closer examination of ascospore morphology, should provide some clarification of these features and clues to its relationship with *Nigrosabulum*, *Bulbithecium*, and *Hapsidospora*. It is likely that developmental studies would show that the ascomata of all four genera would demonstrate similar ontogenetic hallmarks of the hypocrealean centrum.

It is clear that the cleistothecial form has arisen several times within the *Bionectriaceae*, based on available phylogenies (Rehner & Samuels 1995; Suh & Blackwell 1999; Rossman et al. 2001), and appears to parallel a tendency towards the coprophilous habit, which is otherwise rare in the *Hypocreales* (Samuels & Blackwell 2001). This congruence in form and life style is illustrated by most of the astomous genera formerly placed in the *Pseudeurotiaceae* (i.e., *Bulbithecium*, *Hapsidospora*, *Leucosphaerina*, *Mycoarachis*, and *Nigrosabulum*) (Malloch & Cain 1970; von Arx 1987; Udagawa & Muroi 1990), most isolates of which have been obtained from dung. Species of *Emericellopsis* are an exception to this pattern because they are associated with wet and waterlogged soils or muds in aquatic or marine environments rather than dung (Zuccaro et al. 2004).

A second lineage includes *Heleococcum* (Udagawa et al. 1995) a cleistothecial genus, which is apparently closely related to the perithecial *Hydropisphaera* (Rossman et al. 2001), and the somewhat similar genus *Roumegueriella* (Udagawa et al. 1994). These two cleistothecial genera have the light to brightly coloured peridial tissues typical of the order, and are also relatively distant to the group of cleistothecial taxa described above. *Heleococcum* and *Roumegueriella* accommodate versatile saprobes that occur on soil, wood and sometimes dung. Unfortunately, detailed structural data are not available for either taxon and neither has been studied developmentally (Udagawa et al. 1994, 1995; Rossman et al. 1999).

Rehner & Samuels (1995) refer to ostiolar development as relatively facile among the *Hypocreales* and indeed astomy could result merely from an interruption of the normal growth of the apical meristem that generates the cells of the neck, and eventually the ostiole. The cleistothecial habit suggests also that the ascomata themselves could function as propagules. The frequent observation of germinated ascospores within the abundant melanized and grit-like ascomata in *N. globosum* may indicate that these reproductive units not only have protection from uv and desiccation but also are primed and ready to initiate growth when transferred by some unknown means to fresh dung. The relative impermeability of this layer was evident during our attempts to fix whole ascomata for TEM. The heavily melanized layer was an effective barrier to the infiltration process.

The proclivity of the *Hypocreales* for the production of biologically active compounds, (e.g., trichothecenes, fumonisins and short chain antimicrobial polypeptides) (Samuels & Blackwell 2001) could presumably suit

their establishment on dung by discouraging the growth of competing bacteria and fungi. *Nigrosabulum globosum* produces two such peptides, pseudodestruxins A and B (Che et al. 2001) and other antimicrobial compounds have been identified from partially characterized isolates assigned to the genus (Cameron et al. 1974; Murao et al. 1987).

In summary, even though the mature ascoma of *N. globosum* offers few overt morphological clues indicating an affiliation with the *Hypocreales*, its developmental characteristics are fully comparable with perithecial representatives of the order. Stages of development include the formation of a gel-filled centrum, which is replaced by peridial tissue, apically derived paraphyses and finally an ascogenous system that displaces the interascal cells. In the absence of the meristem, which would otherwise form the neck and ostiole, clavate to globose asci remain trapped within the confines of the ascoma wall and deliquesce as the centrum fills with maturing ascospores. Ascospores are not smooth but ornamented with a honeycomb-like reticulum and often have one or two small protuberances indicating the origin of germtubes that eventually elongate and grow into the peridial and stromatal tissues making up the ascoma wall. The darkly pigmented and indehiscent nature of the mature ascomata are interpreted as adaptations to the coprophilous lifestyle, a rare phenomenon among the *Bionectriaceae*.

In their recent critique of the value of the cleistothecium in ascomycete systematics, Stchigel & Guarro (2007) state that this type of ascoma is a poor taxonomic character above the rank of genus. This maxim may be true when the character gets only cursory consideration or is poorly known. We contend that detailed observations of anatomical and developmental features associated with

cleistothecial ascomata can yield a wealth of information especially when applied in conjunction with phylogenetic data from DNA analyses. Further detailed and comparative studies of ascoma structure will serve to strengthen hypotheses concerning the evolution of form in the ascomycetes and the intricacies of their taxonomic relationships.

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Figs 3.1- 3.8. Ascoma development and early centrum structure in *Nigrosabulum globosum* (UAMH 4433).

3.1. Light micrograph showing a globose ascospore that germinated with 2 germ tubes on agar surface. Unstained. Bar = 12 μm.

3.2. Thin section showing ascoma initials consisting of sessile to stalked, globose lateral branches on prostrate vegetative hyphae. Bar = 7 μm.

3.3. Thin section showing initials (antheridia) (arrowheads) encircled by swollen septate hyphae (ascogonia) (arrows) forming lateral buds. Bar = 7 μm.

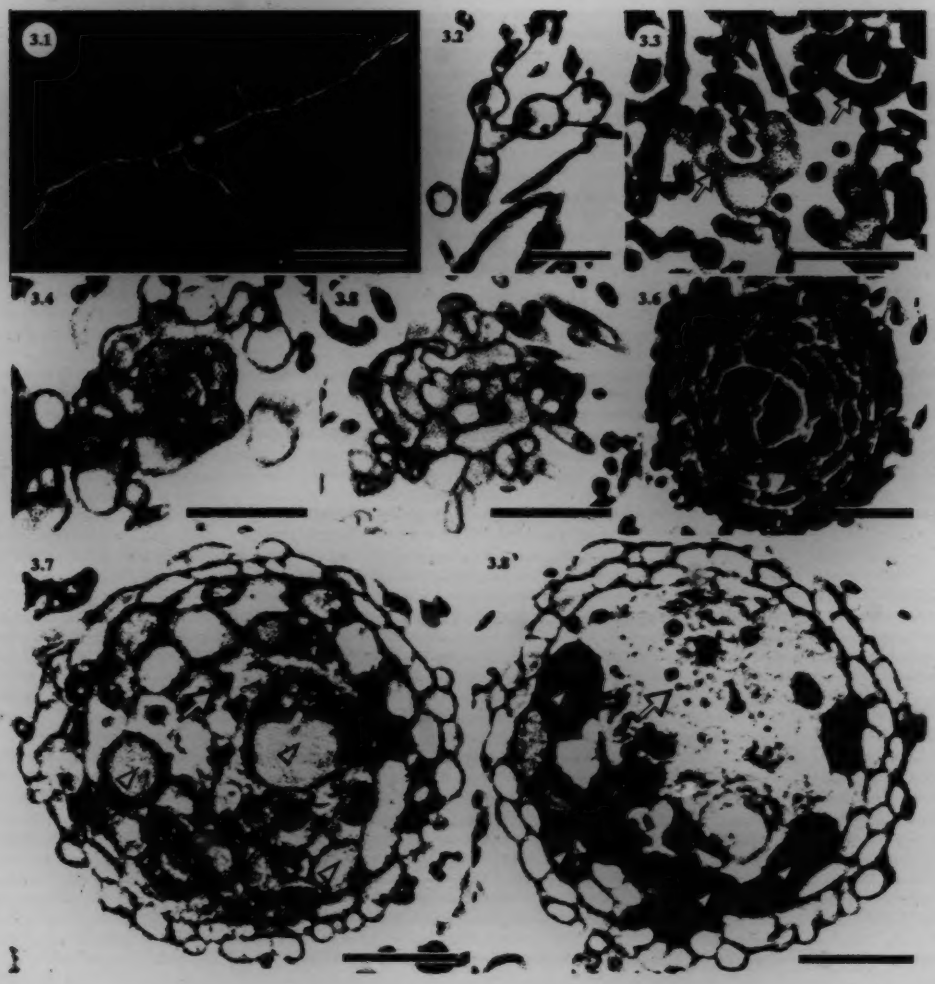
3.4. Thin section showing swollen hyphae branching and over arching the enlarging primordium. Bar = 7 μm.

3.5. Thin section showing broad hyphae arising from vegetative mycelium that are being incorporated into the outer layers of the primordium. Bar = 7 μm.

3.6. Thin section showing coiled gametangia in the center of the young ascoma, surrounded by several layers of pseudoparenchyma.
Bar = 7 μm.

3.7. Thin section through developing ascoma showing thick-walled melanized cells of the peridium surrounding thin-walled cells of centrum that are vacuolated and deliquescing (arrowheads), leaving an amorphous gel containing numerous lipid globules (arrow). Bar = 10 μm.

3.8. Thin section through developing ascoma showing thick-walled melanized cells of the peridium surrounding thin-walled cells of centrum that are deliquescing, leaving an amorphous gel containing numerous lipid globules (arrow). Note the intact, densely cytoplasmic cells on one side of the centrum (arrowheads). Bar = 10 μm.


3.1
3.2
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3.7
3.8

Figs 3.9 - 3.13. Centrum development and ascosporogenesis in *Nigrosabulum globosum* (UAMH 4433).

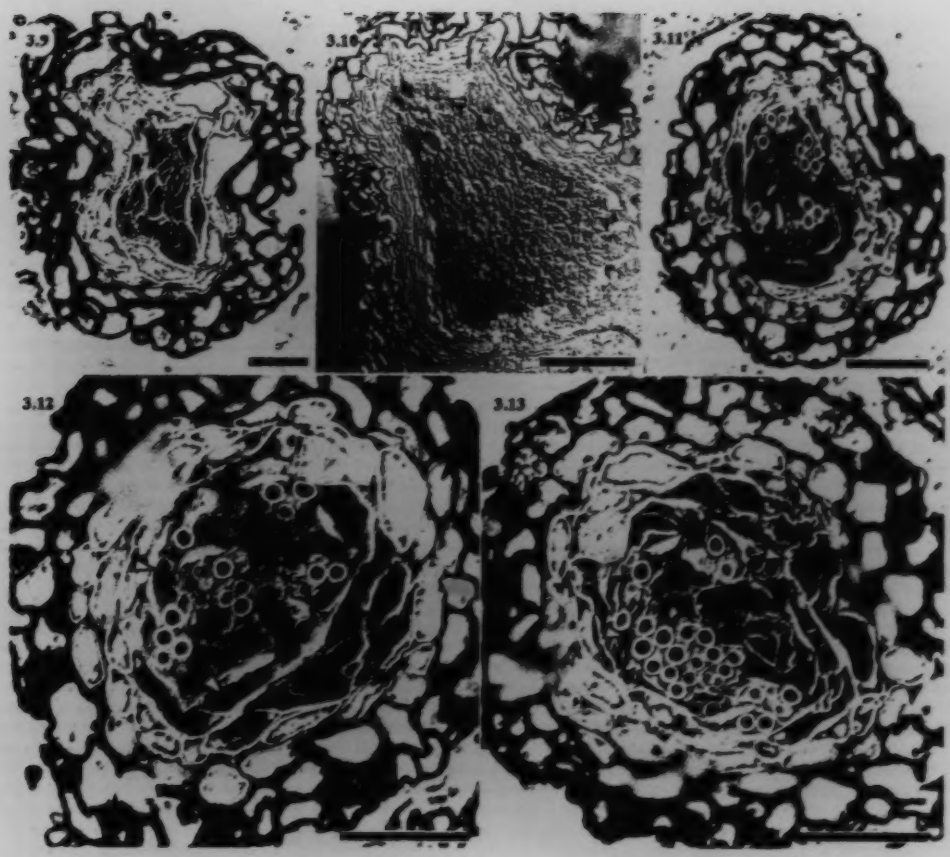
3.9. Thin section through young ascoma showing the development of the bi-layered ascoma wall. Thick-walled hyaline branching hyphae are forming a prosenchyma lining the melanized outer layer of the wall, interpreted, respectively, as the true peridium, and the surrounding stroma. A second prosenchyma, composed of thin-walled cells filling the centrum, is beginning to develop. Bar = 10 μm.

3.10. Thin section through young ascoma showing the development of the bi-layered ascoma wall at a later stage. Fully formed prosenchyma of thin-walled cells are filling the centrum. Bar = 10 μm.

3.11. Thin section through ascoma showing two wall layers, and centrum prosenchyma. Asci are growing up through centrum prosenchyma. Bar = 20 μm.

3.12. Thin section through ascoma showing two wall layers, and centrum prosenchyma. Note some asci are clavate (arrowhead). Bar = 20 μm.

3.13. Thin section through ascoma showing two wall layers, and centrum prosenchyma. Note that some asci are globose to subglobose in shape (arrowheads). Bar = 20 μm.


3.9
3.10
3.11
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3.13

Figs 3.14-3.19. Ascospores of *Nigrosabulum globsum* (UAMH 4433).

3.14. Scanning electron micrograph of ascospores with honeycomb-like network of ridges on the surface. Some spores have germinated and formed germ tubes (arrowheads). Arrow indicates remnants of centrum pseudoparenchyma. Bar = 7 μm.

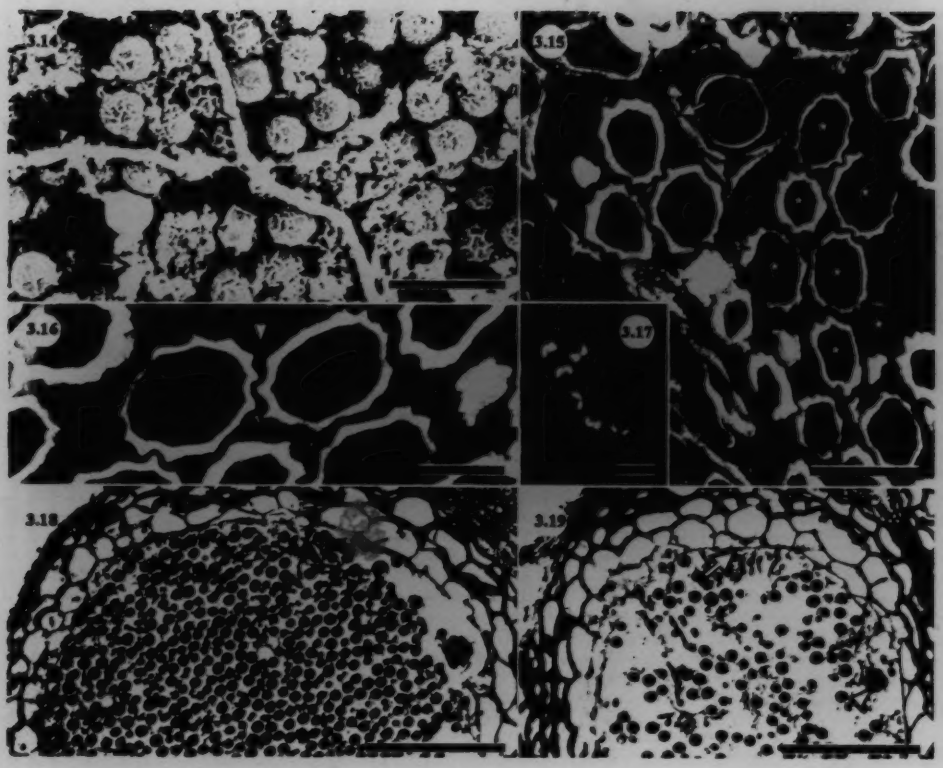
3.15. Transmission electron micrograph showing ascospores with thick electron lucent wall and osmiophilic contents. Spore wall has network of ridges that interdigitate between adjacent ascospores. Note remnants of ascus wall (arrow) and biseriate arrangement of ascospores (stars). Bar = 5 μm.

3.16. Transmission electron micrograph showing ascospores with thick electron lucent wall and osmiophilic contents. Spore wall has network of ridges that interdigitate between adjacent ascospores. Note: remnants of spore delimiting membrane (arrowhead). Bar = 1.7 μm.

3.17. Light micrograph showing binucleate ascospores stained with Hoechst. Both nuclei positioned towards one side of the spore. Bar = 7 μm.

3.18. Light micrograph of thin section through mature ascoma showing that the hyaline peridium lining the melanized stroma, centrum pseudoparenchyma, and asci have evanesced. Note ascospores appear unordered in the centrum. Bar = 25 μm.

3.19. Light micrograph of thin section through mature ascoma showing that the hyaline peridium lining the melanized stroma, centrum pseudoparenchyma, and asci have evanesced. Ascospores have germinated, and germ tubes are penetrating the peridium (arrow). Note structures between spores are remnants of centrum pseudoparenchyma (arrowhead). Bar = 25 μm.


3.14
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Chapter 4

Conclusion

My work shows that previously overlooked centrum characteristics, examined by careful anatomical analysis during their development, can reveal fundamentally important characters that are phylogenetically informative and useful in testing hypotheses derived from DNA sequence comparisons. In this thesis, I describe ascoma development in two unrelated but morphologically and ecologically similar cleistothecial fungi. *Pleuroascus nicholsonii* is a cleistothecial representative of the *Leotiomycetes*, a group in which the apothecium is the usual type of ascoma. *Nigrosabulum globosum* is a cleistothecial representative of the *Sordariomycetes*, a group in which the perithecium, with or without a stroma, is the usual type of ascoma. Until relatively recently, both genera were placed in the *Pseudeurotiaceae*, a cleistothecial family defined on the basis of crude characteristics of the peridium and centrum. Based on DNA sequence comparisons, *Nigrosabulum globosum* is now considered a member of the *Bionectriaceae* of the *Hypocreales*, but an appropriate family and order for *Pleuroascus nicholsonii* within the *Leotiomycetes* is still unknown.

I proposed that the cleistothecia of *Pleuroascus nicholsonii* and *Nigrosabulum globosum* represented neotenous versions of the developmental trajectories, normally found in their respective lineages, that had been interrupted at a cleistohymenial stage. In apothecial lineages, ascogenous tissue is initially enclosed in excipular tissue, but as development proceeds a small central opening develops on the upper surface that allows the excipulum to reflex and fully expose the hymenial tissue. In perithecial lineages, ascogenous

tissue is similarly enclosed by peridial tissue early in development. Later the hymenium is partially exposed by the development of a neck and ostiole formed by an apical meristem. Ostensibly, the unexposed hymenium of a juvenile stage was selected as an evolutionary response to changes in habitat and spore dispersal mechanism.

Both *P. nicholsonii* and *N. globosum* represent lineages in which plants, living or dead are the most common habitat, but these species differ from most in being coprophilous. I contend that the unique challenges posed by this habitat for reproduction and dispersal led to the selection of cleistocarpous forms of their respective apothecial and perithecial ancestors. My ontogenetic data have contributed to the explanation of the possible evolutionary steps involved in the acquisition or loss of various characters and help explain the origins of certain lifestyles (e.g., coprophily).

Coprophily has evolved independently in numerous lineages, and fungi inhabiting dung exhibit a similar spectrum of characteristics including increased size and melanization of propagules that can be single spores or spores aggregated in some way, e.g., as in sporangia, and a movement away from wind-dispersal, which is too random to be effective for reaching discontinuous and island-like dung piles. Instead, coprophilous fungi often have a forcible gun-like mechanism that actively propels sticky propagules away from the habitat as it deteriorates and onto surrounding vegetation (common in fungi that require gut passage through herbivores), or modifications that improve potential for being carried away by small animals, such as arthropods. Over the past five decades there have been repeated suggestions that evolution toward a coprophilous habit in ascomycetous fungi would include a trend towards the

modification of open ascomata with forcibly discharging asci, to the cleistothecial form with non-discharging asci (e.g., Cain 1956, Benny & Kimbrough 1980; Malloch 1981; Samuels & Blackwell 2001). Such a modification would also include the loss of the mechanism giving rise to the open excipulum and ostiole, a non-layered hymenium, a loss or reduction of paraphyses, asci changing from cylindrical or clavate to globose and evanescent, and a probable change in ascospore shape from ellipsoid, which allows easy firing from an ascus, to globose (Cain 1956; Benny & Kimbrough 1980; Malloch 1981; Samuels & Blackwell 2001).

However, *P. nicholsonii* and *N. globosum* differ from this model in some respects. For example, in *P. nicholsonii*, paraphyses persist as interascal elements, probably because they play a role in the fitness by providing support for the peridium during ascospore release. In *N. globosum*, the pseudoparenchyma derived from the apical paraphyses still have an important role in development and expansion of the ascoma, as they do in perithecial relatives.

In addition, I point out that although asci in both taxa are superficially globose, there are characteristics that ally these taxa with their respective relatives and differentiate them from the morphology and development of true, or much more ancient, cleistothecial taxa such as represented by the *Eurotiales*. In *P. nicholsonii* ascospores are formed in a twisted, but uniseriate arrangement within the spore delimiting membrane, but the ascus wall itself is globose. In *N. globosum*, ascus morphology is dependent on time of development. First formed asci are clavate, later formed asci are globose to subglobose because of space restrictions within the centrum. Ascospore arrangement, i.e., uniseriate, or

biseriate, is otherwise similar to that found in close perithecial relatives, i.e., *Hydropisphaera peziza* or *Bionectria ochroleuca* (Hanlin 1961; 1963).

Samuels & Blackwell (2001) surmised that there would be a loss of stroma development in cleistothecial taxa derived from pyrenomycetous (i.e., perithecial) lineages, but this hypothesis is not supported here. Instead, I propose that the bi-layered peridium of *N. globosum*, and possibly other cleistothecial members of the *Bionectriaceae*, consists of an outer layer that represents modified, or possibly reduced, stromatal tissue. In *N. globosum* the outer layer of the peridium is thick walled, melanized and separated by time of development from the inner hyaline layer, which I propose is the true peridium. This is supported by the observation that many members of the *Hypocreales* have lightly to brightly coloured peridia. This model contrasts with the bi-layered peridium of *P. nicholsonii*, in which the outer melanized layer matures as an integral part of the peridium, which appears to have been derived from excipular tissue of its apothecial forerunner.

Both *P. nicholsonii* and *N. globosum* produce ascospores in rapidly maturing cleistothecia. Abundant ascomata are formed within 2 weeks for both species. In both taxa, asci evanesce early in development, releasing ascospores into the centrum. Ascospores in each are hyaline rather than melanized, lack adhesive material, and germinate readily, i.e., do not require gut passage. It seems likely that the ascomata themselves are the dispersal units in these species. If so, they would need some method of being transferred from exhausted to fresh habitat. I suspect that the helical appendages of *P. nicholsonii* in combination with the peridium that splits between intercellular grooves, and the grit-like nature of the ascomata of *N. globosum* with the germination of

ascospores in the centrum are related to specific dispersal strategies, but the mechanisms involved are unknown.

My research also demonstrates that previously overlooked structural details can be used to define the limits of taxa (e.g., at the genus level), that otherwise appear enigmatic in phylogenetic trees. For example, my detailed morphological studies suggest that *Pleuroascus* and *Connersia* may be congeneric, a relationship also indicated to some extent by their position relative to each other in molecular phylogenies. A similar close relationship exists between *Nigrosabulum* and *Hapsidospora*, and perhaps *Bulbithecium*.

This thesis also emphasises the importance of developmental studies for providing taxonomic characters in order to clarify higher level relationships. Developmental studies among the cleistothecial and perithecial *Bionectriaceae* might assist in resolving the lineages within this group. Morphological details associated with ascoma development would also be helpful in determining the appropriate family and order for *Pleuroascus* and its relatives, which at the moment stand as *incertae sedis* (Erikson et al. 2001), and in resolving relationships of other taxa currently marooned among the "residual Pseudeurotiaceae" (Suh & Blackwell 1999).

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Appendix A

ITS rDNA sequence data for *Connersia rilstonii* UAMH 10834

644 basepairs

CTGGATAGGCTATTATGACGCTCGAACAGGCATGCCCCTCGGAATAC
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TGTTTAGGGTCTCGCGATCCCCCCCGCTGGGTCTCCCCAGCGGACCGA
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GAACGTTCCCCGTAAGCTCGCGCTCTTAGGGGCTTCGCTGCGGATCA
TCCATTGTCCATCCAGAGGGGTTCTGACTGTACCCCGCGCTGTTAAC
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AGGAGGTCCCCGCACTTCGATTATGTTGCCGGCGGCCACCGAGGCAG
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Appendix B

SSU rDNA sequence data for *Connersia rilstonii* UAMH 10834

1528 base pairs

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AGGCTCTGCTCCGGACAGTAGCCCCCCTCCGGGGGCGGTGGTGGCCC
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Appendix C

ITS rDNA sequence data for *Nigrosabulum globosum* UAMH 4433

719 base pairs

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Appendix D

SSU rDNA sequence data for *Nigrosabulum globosum* UAMH 4433

1705 base pairs

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Appendix E

ITS rDNA sequence data for *Nigrosabulum globosum* UAMH 6868

515 base pairs

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TCAGAATATCCCACTGTAAAGACAAGAGTTTGGGTCCCTCGGCGGGC
GCCTGGTTCCGGGGCGCGGCAGCCGCTAGGGGCCGCCGCGCGCCCG
GGGCGATCCCCGCCGAAGCAACGATGGTATGTTCACAAAGGTTTGGG
AGTTCTGTAACTCAATAATGATCCCTCCGCTGGTTCACCAACGGAGA
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Appendix F

SSU rDNA sequence data for *Nigrosabulum globosum* UAMH 6868

1678 base pairs

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Appendix G

ITS rDNA sequence data for *Pleuroascus nicholsonii* UAMH 3979

413 basepairs

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Appendix H

SSU rDNA sequence data for *Pleuroascus nicholsonii* UAMH 3979

1709 base pairs

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CCGGGGCTGGGAAGCTGTTCAAACTTGGTCATTTAGAGGAAGTAAAA
GTCGTAACAAGGTTTCCGTA

Appendix I

Glossary of terms

This glossary contains terms used in this thesis. Definitions are modified from Currah 2008, and Kirk et al. 2001.

Anamorph - the asexual, or imperfect, state of a fungus.

Annelidic- type of conidiogenesis in which conidia are produced in a serial, basipetal manner by a flask-shaped conidiogenous cell that elongates slightly with successive formation of conidia (annellide).

Antheridium (**-ia**, pl.) - male gametangium, a cell that donates a nucleus during sexual reproduction.

Aporate - lacking a germ slit or pore (thin, differentiated areas of the spore wall).

Apothecium (**-ia**, pl.) - a cup-shaped or saucer-shaped ascocarp, with an exposed hymenium at maturity.

Arthroconidia - conidia that are formed through the conversion of a preexisting, determinate hypha into a conidium or conidia.

Ascogonium (**-ia**, pl.) - female gametangium, a cell that is fertilized during sexual reproduction.

Ascoma (**-ta**, pl.) - a fruit body of ascomycetes in which asci are formed. Also known as an ascocarp.

Ascospore - meiotic propagules of ascomycetes

Ascus (**-i**, pl.) - a sac-like cell in which ascospores develop after karyogamy and meiosis.

Capillitium (**-ia**, pl.) - collective term for the sterile filamentous hyphae among the spores of gasteromycetes.

Catenate - in chains, or an end-to-end series.

Centrum - the central cavity of an ascoma including asci and paraphyses, and their relationship to the ascoma wall.

Clavate - widened at the distal end, or club-shaped.

Cleistothecium (**-ia**, pl.) - closed, or ball-shaped ascoma, with hymenial tissues enclosed at maturity.

Cleistohymenium - hymenial tissues are enclosed in a cavity surrounded by sterile tissue. Used in reference to development, particularly in apothecial ascomycetes.

Conglobate - round, compact mass.

Decussate - arranged in a crosswise formation.

Deliquescent - dissolving.

Discomycete - general, non-taxonomic term referring to any apothecial ascomycete.

Excipulum (**-a**, pl.) - the sterile tissue forming the ascomatal wall in apothecial and perithecial taxa.

Fusiform - tapering at each end, spindle-shaped

Gametangium (**-ia**, pl.) - cell containing gametes.

Gamete - uninucleate haploid cells with the sole function of fusing with another to form a zygote.

Gastroid - a closed or ball-shaped form of fruit body in which the fungus has lost active meiospore release, and the spores are retained within a central cavity (or "gut"-like structure), such as a puffball.

Gastromycete - general, non-taxonomic term referring to gastroid basdiomycetes.

Globose - globe shaped, spherical.

Gymnothecium (**-ia**, pl.) - a type of globose, non-ostiolate ascoma in which the peridium is composed of a loose network of bramble-like hyphae.

Hyaline - transparent, or nearly so, or colourless.

Hymenium (**-ia**, pl.) - the fertile, spore bearing layer of an ascoma

Initial - the first indications of the formation of an ascoma, typically through the formation of gametangia, or hyphal coils.

Inner membrane - membrane of spore delimiting membrane system that becomes the plasma membrane of the ascospore.

Inoperculate (discomycetes) - lineage of apothecial taxa in which asci lack a specialized operculum or cap that opens to permit spore discharge. Spores are typically liberated through a split or pore in the ascus.

Locule - cavity, especially in a stroma.

Melanized - pigmented dark brown to black.

Navicular - boat-shaped.

Operculate (discomycetes) - lineage of apothecial taxa in which asci develop with a specialized apical cap or lid that opens to permit spore discharge.

Ostiole - a pore-like opening at the apex of the neck of a perithecium.

Paraphysis (**-es**, pl.) - Sterile, branched or unbranched filaments ordered parallel to cylindrical to clavate asci in a hymenial layer.

Peridium (**-ia**, pl.) - ascomatal wall, typically refers to cleistothecial taxa.

Periphysis (**-es**, pl.) - sterile hyphal elements inside or near the ostiole of a perithecium.

Perithecium (**-ia**, pl.) - a flask-shaped or subglobose ostiolate ascoma, with a partially enclosed hymenium at maturity.

Petaloid - radially arranged, like the petals of a radially symmetrical flower.

Phialidic - type of conidiogenesis in which conidia are produced in a serial, basipetal manner by a flask-shaped conidiogenous cell (phialide).

Phialoconidium (**-ia**, pl.) - conidium produced by a phialide.

Plectomycete - general, non-taxonomic term referring to cleistothecial ascomata with globose, evanescent, and irregularly arranged asci.

Primordium (**-ia**, pl.) - early stage of ascoma formation. Tissue has not yet differentiated into mature structures.

Prosenchyma - type of fungal tissue formed by closely packed hyphae that are still recognizable as such.

Prototunicate - refers to asci that liberate ascospoes by wall lysis rather than through an opening at the tip.

Pseudoparenchyma - a type of fungal tissue formed by hyphae that has a cellular, parenchymatous appearance.

Pseudothecium (**-ia**, pl.) - ascostromatic ascoma having asci in unwalled locules.

Reticulate - net-like.

Spore investing membrane - the outer membrane of a double membrane system of ascospore formation. Also known as spore-delimiting membrane or ascus vesicle.

Stroma - loose to consolidated layer of vegetative hyphae within which ascomata can form.

Squamulose - resembling scales in form and arrangement.

Subglobose - not quite spherical.

Subiculum - vegetative mycelial growth underlying the fruit bodies of some fungi.

Sympodial - the main axis being made up of successive secondary axes.

Teleomorph - the sexual, perfect state of a fungus.

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